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Characteristics of Fps1-Dependent and -Independent Glycerol Transport in *Saccharomyces cerevisiae*

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Eadie-Hofstee plots of glycerol uptake in wild-type *Saccharomyces cerevisiae* W303-1A grown on glucose showed the presence of both saturable transport and simple diffusion, whereas an *fps1*Δ mutant displayed only simple diffusion. Transformation of the *fps1*Δ mutant with the *glpF* gene, which encodes glycerol transport in *Escherichia coli*, restored biphasic transport kinetics. Yeast extract–peptone–dextrose-grown wild-type cells had a higher passive diffusion constant than the *fps1*Δ mutant, and ethanol enhanced the rate of proton diffusion to a greater extent in the wild type than in the *fps1*Δ mutant. In addition, the lipid fraction of the *fps1*Δ mutant contained a lower percentage of phospholipids and a higher percentage of glycolipids than that of the wild type. Fps1p, therefore, may be involved in the regulation of lipid metabolism in *S. cerevisiae*, affecting membrane permeability in addition to fulfilling its specific role in glycerol transport. Simultaneous uptake of glycerol and protons occurred in both glycerol- and ethanol-grown wild-type and *fps1*Δ cells and resulted in the accumulation of glycerol at an inside-to-outside ratio of 12:1 to 15:1. Carbonyl cyanide *m*-chlorophenylhydrazone prevented glycerol accumulation in both strains and abolished transport in the *fps1*Δ mutant grown on ethanol. Likewise, 2,4-dinitrophenol inhibited transport in glycerol-grown wild-type cells. These results indicate the presence of an Fps1p-dependent facilitated diffusion system in glucose-grown cells and an Fps1p-independent proton symport system in derepressed cells.

Glycerol crosses all biological membranes by passive diffusion due to its lipophilic nature. In addition, specific transport proteins are frequently produced by microorganisms, resulting in more rapid transport of glycerol across the membrane. Active glycerol transport systems requiring the expenditure of metabolic energy have been identified in *Zygosaccharomyces rouxii*, *Debaryomyces hansenii* and *Pichia sorbitophila* (21, 23, 36), whereas glycerol crosses the *Escherichia coli* cytoplasmic membrane via a proteinaceous pore mechanism which is encoded by *glpF* (15).

It has been assumed that glycerol is taken up by *Saccharomyces cerevisiae* by passive diffusion only. Recently *FPS1*, which encodes a protein belonging to the MIP family, has been shown to affect the movement of glycerol across the membrane of *S. cerevisiae* (24). The *FPS1* gene was isolated as a multicopy suppressor of the growth defect on fermentable sugars of a yeast *fdp1* (*FDP1* is also known as *CIF1* and *GGS1*) mutant (33). Fps1p seems to play an important role in glycerol efflux, since mutants lacking *FPS1* fail to rapidly release excess glycerol when hyperosmotic stress is relieved and during glycerol overproduction (reference 24 and unpublished results).

The MIP family is a group of channel proteins present in organisms ranging from bacteria to humans (28). Most of these proteins are around 250 to 280 amino acids long and consist of six membrane-spanning segments. Fps1p differs from most members of the MIP family by having long hydrophilic N- and C-terminal extensions (28), the functions of which have not yet been elucidated.

The membrane lipid composition of an organism can affect the rate of both passive diffusion and protein-facilitated transport. Integral membrane proteins usually require specific lipids for optimal activity and are inhibited by other lipid species (16). The activities of membrane proteins are also sensitive to lipid bilayer dynamics and physical-chemical state, which is in turn determined by the lipid composition of the membrane (25, 34).

In this work, glycerol transport in *S. cerevisiae* has been characterized in terms of kinetics and the number and nature of transport systems present. In addition, the effect of deletion of *FPS1* on lipid composition was also investigated. The results show that both an Fps1p-dependent facilitated transport mechanism and an active transport system independent of Fps1p are operative in *S. cerevisiae*. Observations that suggest a role for Fps1p in the control of passive diffusion are also discussed.

MATERIALS AND METHODS

Yeast strains. Congenic strains of *S. cerevisiae* W303-1A were used (31). The construction of the *fps1*Δ mutant and the YEp*FPS1* multicopy plasmid were previously described by Van Aelst et al. (33). The YEp*glpF* multicopy plasmid, which expresses the *E. coli* glycerol facilitator gene *glpF* under the control of the strong yeast phosphoglycerate kinase (*PGK1*) promoter, has been described by Luyten et al. (24).

Cultivation. Strains were grown at 30°C for 12 h on a rotary shaker at 180 rpm in 500-ml Erlenmeyer shake flasks containing 100 or 200 ml of the appropriate medium. In assays for active uptake determination, cells were collected in mid-exponential phase (A_{640} of 0.3 to 0.4). YEPD medium consisted of (per liter) 20 g of peptone, 10 g of yeast extract and 20 g of glucose. YEPG and YEPE were similarly constituted but contained 20 g of glycerol or ethanol · liter^{-1}, respectively, as a carbon source. Mineral medium (MM)-glucose, MM-glycerol-glucose,

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and MM-ethanol liquid media contained minerals, as previously described (35), and 20 g of glucose, glycerol, or ethanol · liter^{-1}, respectively, as the main substrate. MM-glycerol-glucose contained, in addition to glycerol, 2 g of glucose · liter^{-1} as a "starter" substrate.

Glycerol accumulation. Accumulation of glycerol in ethanol- and glycerol-grown cells was determined as previously described (21) by using [^{14}C]glycerol (156 mCi · mmol^{-1}, 50% ethanolic solution; Amersham). Intracellular and extracellular glycerol concentrations were measured with a Gilson high-performance liquid chromatograph with a Merck Polyspher OA KC (catalog no. 51270) column maintained at 50°C. Sulfuric acid (0.05 N in ultrapure water) served as the mobile phase at a flow rate of 0.5 ml · min^{-1}. The intracellular volume was determined by using tritiated water and [^{14}C]inulin, as previously described (12, 29).

Glycerol and proton transport rates. For determination of initial transport rates, exponential-phase cells were harvested by centrifugation, washed twice with cold distilled water, and resuspended in ice-cold distilled water or morpholineethanesulfonic acid buffer. Changes in the extracellular pH upon glycerol addition were measured as previously described (23). Uptake of [^{14}C]glycerol by YEPD-grown cells was measured by exposing cell suspensions to [^{14}C]glycerol (with variable specific activities ranging from 30 to 750 mCi · mmol^{-1}; Amersham) for 10 s, stopping the reaction by dilution with 5 ml of ice-cold water, filtering, and counting the radioactivity on the filter in a scintillation counter. All other assays were performed as described before (22). Kinetic constants were derived by Eadie-Hofstee plots and confirmed with Lineweaver-Burk plots. Data displaying biphasic kinetics were further analyzed by computer-assisted iteration (SigmaPlot; Jandel Scientific, San Rafael, Calif.) using the following equation for passive diffusion and mediated uptake components acting simultaneously: $v = -K_m(v/S) + V_{max} + D(K_m + S)$, where D is the passive diffusion coefficient. Enhancement of the passive proton diffusion constant by ethanol was calculated from the slopes of plots of final pH (pH_f) versus ethanol concentration, according to the following relationship: $pH_f = \log_{10}C_o - \log_{10}k_p + 2.303kx$, where k_p is the rate of active proton extrusion (mol · s^{-1}), x is the ethanol concentration, C_O is the value of the proton diffusion constant in the absence of ethanol, and k is the proton diffusion enhancement constant (17).

Analytical methods. Dry mass was determined gravimetrically in triplicate. Lipid analysis was done on YEPD-grown cells harvested in the late exponential phase (dry biomass ≈ 1.3 g · liter^{-1}), as previously described (19).

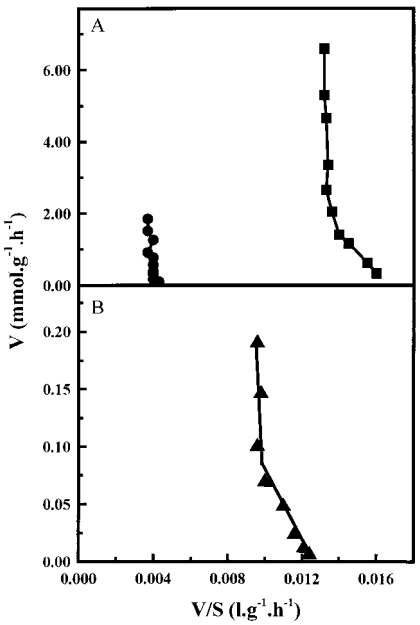

FIG. 1. Eadie-Hofstee plots of initial uptake rates of [^{14}C]glycerol at pH 5 and 30°C by wild-type W303-1A (■) and W303-1A *fps1*Δ mutant (●) strains grown in YEPD medium (A) and initial uptake rates of [^{14}C]glycerol at pH 5 and 30°C by the W303-1A *fps1*Δ mutant transformed with YEp*glpF* and grown in yeast nitrogen base (YNB) (B).

RESULTS

***FPS1P* involvement in glycerol transport.** Eadie-Hofstee plots of glycerol uptake in the wild type grown on YEPD showed biphasic kinetics (Fig. 1A). Kinetics at low glycerol concentrations were compatible with a saturable uptake system, and those at higher glycerol concentrations were compatible with passive diffusion. Similar results were obtained with glycerol transport in *E. coli*, in which the *glpF* gene encodes glycerol transport (2). Passive diffusion only was evident in the *fps1*Δ mutant (Fig. 1A). The corresponding kinetic parameters are presented in Table 1. Wild-type cells grown in MM and harvested during exponential phase did not present saturable kinetics for glycerol uptake. However, when harvested in early stationary phase (A_{640}, 0.6 to 0.8), wild-type cells displayed saturable transport, with a K_m of 26 mM and a V_{max} of 233 μmol · h^{-1} · g^{-1}. No saturable kinetics were found in *fps1*Δ mutant cells harvested either in exponential or early stationary growth phase, indicating that this system was dependent upon Fps1p. The reasons for the absence of the saturable system in early exponential cells and the lower affinity of this system in cells grown in MM compared with YEPD-grown cells (Table 1) are not evident but may be due to a regulatory effect.

The passive diffusion coefficient was 0.013 ± (6 × 10^{-4}) liters · g^{-1} · h^{-1} (mean ± standard deviation) in the wild type and 0.004 ± (2 × 10^{-4}) liters · g^{-1} · h^{-1} in the *fps1*Δ mutant in YEPD medium (Table 1). This implies a function for *FPS1* in the control of passive diffusion in addition to its role in glycerol transport. When grown in MM, the deletion mutant and the wild type had similar diffusion coefficients (Table 1), indicating that medium composition could also affect the kinetics of simple diffusion. It has been reported that medium composition can affect fatty acid synthesis (14) and membrane structure (39) in yeasts, and these changes may affect membrane function and hence diffusion rates of molecules through the lipid bilayer.

Expression of GlpF in *S. cerevisiae*. Strong molecular homology exists between Fps1p and the GlpF glycerol facilitator of *E. coli* (24). In addition, transformation of the *fps1*Δ mutant with *glpF* partially substitutes for yeast Fps1p functions (24). It was of interest, therefore, to determine the transport kinetics of the *E. coli* facilitator expressed in *S. cerevisiae*. Eadie-Hofstee plots of transport kinetics of the YEp*GlpF* transformants showed, like the wild type, biphasic transport (Fig. 1), reflecting the simultaneous operation of passive diffusion and facilitated transport. It can be concluded, therefore, that *glpF* is functionally expressed in *S. cerevisiae*. The *glpF* transformant, however, had a K_m of 0.04 mM, which is about 100-fold lower than that of the wild type (Table 1). Similarly, glycerol transport in *E. coli* has a 1,000-fold-lower K_m than in wild-type *S. cerevisiae* (37) (Table 1).

Energetics of Fps1p-dependent glycerol transport. The effect of various metabolic inhibitors on the uptake of 20 mM radioactively labeled glycerol in YEPD-grown wild-type cells was determined in investigating the possibility that Fps1p-facilitated transport was dependent upon metabolic energy. Miconazole (5 μM), 2,4-dinitrophenol (5 mM), diethylstilbestrol (5 mM), and vanadyl sulfate (5 μM) had no effect. In addition, measurement of pH changes during uptake showed no evidence of simultaneous uptake of glycerol and protons in glucose-grown cells of either the wild type or the *fps1*Δ mutant, and the intracellular glycerol concentration did not exceed the diffusion equilibrium. It was concluded, therefore, that Fps1p-dependent transport in glucose-grown exponential-phase cells is facilitated diffusion and not active transport.

Active transport in glycerol- and ethanol-grown cells. In contrast to glucose-grown cells, glycerol uptake in either glycerol- or ethanol-grown cells of both the wild type and the *fps1*Δ mutant was accompanied by the simultaneous uptake of pro-

TABLE 1. Kinetics constants for glycerol transport in *S. cerevisiae* W303-1A

Strain	Growth medium	Kinetics constant[a]			Proton uptake upon glycerol addition
		K_m (mM)	V_{max} (μmol · g^{-1} · h^{-1})	Passive diffusion coefficient (liters · g^{-1} · h^{-1})	
Wild type	MM-glucose	ND*[b]	ND*	0.004 (2)	−
	MM-glycerol-glucose	2.0 ± 0.3 (3)	186.4 ± 41.2 (3)	0.005 (1)	+
	YEPD	5.0 ± 0.3 (5)	100.0 ± 5.0 (5)	0.013 ± (6×10^{-4}) (5)	−
	YEPG	2.5 ± 1.1 (3)	160.9 ± 4.6 (3)	ND	+
	YEPE	2.1 ± 0.4 (3)	253.1 ± 46.3 (3)	0.006 (2)	+
*fps1*Δ	MM-glucose	ND	ND	0.005 (2)	−
	MM-glycerol-glucose	2.7 ± 0.9 (3)	312.4 ± 34.4 (3)	0.005 (1)	+
	YEPD	ND	ND	0.004 ± (2×10^{-4}) (5)	−
	YEPG	3.0 (2)	221.5 (2)	0.006 (2)	+
	YEPE	2.7 ± 0.4 (3)	298.0 (3)	ND	+

[a] Numbers of independent experiments given in parentheses.
[b] ND, no saturable transport or no simple diffusion detected; ND*, not detected in standard assay with mid-exponential-phase cells.

tons (Table 1). The K_m of proton uptake could not be determined accurately due to the strong alkalinization of cell suspensions before glycerol addition. V_{max} values of 148.8 and 77.4 μmol · g^{-1} · h^{-1} were obtained for proton uptake in the wild type and the *fps1*Δ mutant, respectively. Eadie-Hofstee plots of initial uptake rates in glycerol- and ethanol-grown cells of the *fps1*Δ mutant displayed saturable kinetics (Fig. 2). [^{14}C]glycerol was accumulated to inside-to-outside ratios of approximately 12 and 15 in the wild type and the *fps1*Δ mutant, respectively, when grown on either ethanol or glycerol. This accumulation was prevented by the presence of 50 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (results not shown). In addition, 40 μM CCCP severely inhibited glycerol transport in the *fps1*Δ mutant grown on ethanol (Fig. 2B). In the wild type, the extent of the inhibitory effect was dependent upon the glycerol concentration used in the transport assay (Fig. 3). At higher concentrations, at which the contribution of simple diffusion to uptake was significant, CCCP had little effect on the uptake rate. In contrast, at lower substrate concentrations, at which the saturable system was dominant, CCCP inhibition of glycerol transport was as high as 50%. 2,4-Dinitrophenol (5 mM) also inhibited glycerol transport in wild-type glycerol-grown cells by 35% (results not shown). These results clearly indicate the production of an Fps1p-independent proton symport system in *S. cerevisiae* W303-1A cells grown on ethanol or glycerol that is repressed by growth on glucose. This system was detected throughout the exponential growth phase with ethanol as the substrate, but in glycerol-grown cells its induction seemed to be stringently controlled by the physiological condition of the cells. The system was detected only during an "induction window" defined by the time interval between glucose starter depletion and the start of glycerol consumption. The differences in the CCCP inhibition pattern in the wild type and the *fps1*Δ mutant are consistent with the coexistence of the Fps1p-dependent facilitated diffusion and active systems under these conditions. These results fully agree with observations of a different strain of *S. cerevisiae*, which also indicated the presence of an active system subject to gluconeogenic induction (22), and show that this system is independent of Fps1p.

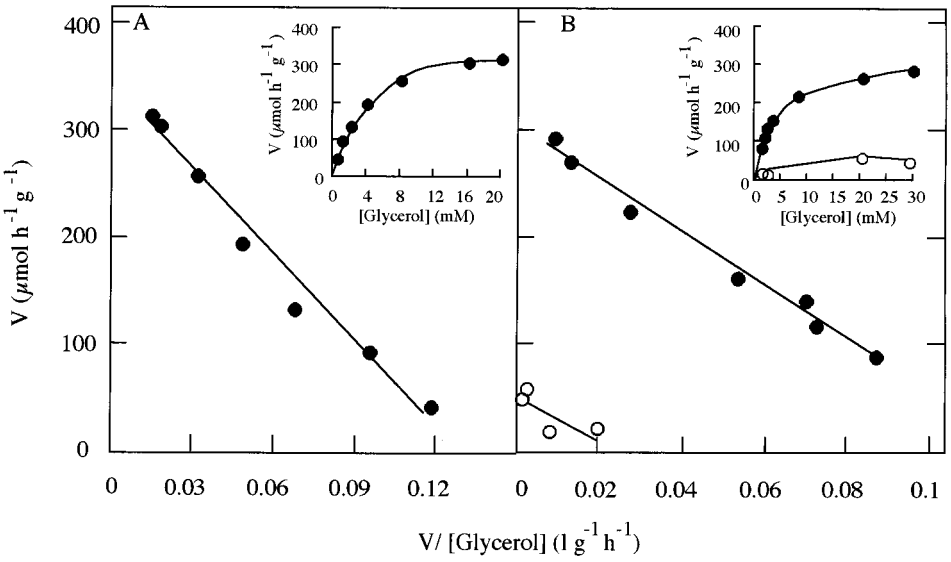

FIG. 2. Eadie-Hofstee plots of initial uptake rates of [^{14}C]glycerol at pH 5.0 and 30°C by the W303-1A *fps1*Δ mutant grown in YEPG (A) and YEPE (B). Assays were performed in the absence (●) and presence (○) of 40 μM CCCP.

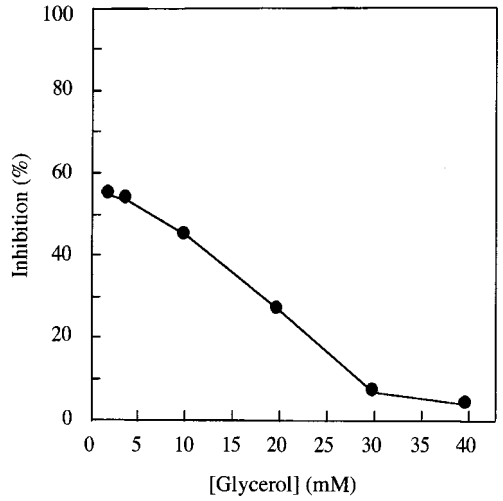

FIG. 3. Effect of 40 μM CCCP on [^{14}C]glycerol uptake by wild-type W303-1A grown in YEPE.

Other effects of Fps1p on membrane functions and on lipid composition. The observation that YEPD-grown wild-type cells had a higher passive diffusion coefficient (0.013 liters · g^{-1} · h^{-1}) for glycerol uptake than the *fps1*Δ mutant (0.004 liters · g^{-1} · h^{-1}) (Table 1) prompted us to further investigate plasma membrane permeability in both strains. To investigate possible differences in general permeability, we determined the ethanol-enhanced proton diffusion constants in both strains. The plots of final pH (pH_f) as a function of ethanol concentration were linear but biphasic in both strains, displaying different slopes above and below 1.36 M ethanol (results not shown). Similar results were previously obtained for *S. cerevisiae* strains as well as a number of other yeasts (18). Below the transition concentration both strains had similar enhancement constants (wild type, 0.23 M^{-1}; *fps1*Δ mutant, 0.30 M^{-1}), but above this concentration substantial differences were observed (wild type, 1.12 M^{-1}; *fps1*Δ mutant, 0.54 M^{-1}). These results indicated *FPS1*-dependent differences in membrane properties between the two strains, with the wild type having greater permeability towards ethanol-enhanced proton diffusion than the *fps1*Δ mutant. This is in agreement with the data on diffusion of glycerol and indicates that Fps1p probably affects membrane structure or composition.

To investigate the possibility that the observed effect of *FPS1* deletion on membrane permeability reflects a role for Fps1p in controlling membrane composition, we determined the effect of *FPS1* deletion on cell lipid composition. The phospholipid and glycolipid fractions of the total lipid of the *fps1*Δ mutant were 25% lower and 62% higher, respectively, than in the wild type (Table 2). There was no significant difference in the sterol composition of these strains (data not shown). These results strengthen the conclusion that Fps1p has, in addition to its function in glycerol transport, a role in determining membrane composition.

TABLE 2. Lipid composition of exponential-phase YEPD-grown wild-type *S. cerevisiae* W303-1A and *fps1*Δ mutant cells[a]

Strain	Lipids in biomass (%)	Lipid composition (%)		
		Neutral lipid	Glycolipid	Phospholipid
Wild type	3.3 (0.2)	64.1 (3.8)	9.2 (0.5)	26.7 (1.6)
*fps1*Δ	4.8 (0.3)	65.2 (3.9)	14.9 (0.9)	19.9 (1.2)

[a] Standard deviations of three independent experiments given in parentheses.

DISCUSSION

The data presented here provide evidence that *S. cerevisiae* produces a constitutively expressed glycerol transport protein which operates by facilitated diffusion. Deletion of *FPS1* abolishes this transport system. These results and the high degree of homology between Fps1p and other members of the MIP family of transport proteins, including several glycerol facilitators, strongly suggest that Fps1p itself is the transport protein. This notion is further supported by the observation that *E. coli* GlpF, expressed in an *fps1*Δ mutant, can partially replace Fps1p for glycerol transport. The possibility that Fps1p acts as a regulator of an unidentified glycerol transport protein, however, cannot be excluded. In this regard, the role of Snf3p in glucose transport in *S. cerevisiae* is worth noting. Early data indicating the dependence of high-affinity glucose transport upon *SNF3* and the high degree of homology between the *SNF3* gene product and known hexose transport proteins led to the conclusion that *SNF3* encoded the high-affinity glucose transport protein (6, 9). Subsequent observations, however, showed that it probably functions as a regulator of glucose transport rather than as a structural transport protein (5). Recently it has been shown that the actual role of Snf3p is in the sensing of low glucose concentrations (11, 26). This may be followed by the initiation of a signaling cascade involving the C terminus, since this domain has been shown to regulate the ability of the yeast to grow at low glucose concentrations (11, 26). It is interesting that Snf3p, like Fps1p, is distinguished from most membrane proteins in its class by exceptionally long amino- and carboxyl-terminal domains (5, 28).

Nonlinear transport kinetics such as those reported here for glycerol transport in wild-type cells are open to widely differing opinions, as illustrated by the controversy surrounding the interpretation of glucose transport kinetics in *S. cerevisiae*. These kinetics have been interpreted as indicative of high- and low-affinity systems by some authors (10), whereas others have suggested that it represents the simultaneous action of a facilitated diffusion system and passive diffusion (13). Yet another possibility is that one system is constitutively expressed but its affinity is regulated by the prevailing glucose concentration (38). We have proposed the biphasic kinetics of glycerol transport to be a model for a facilitated diffusion system and a passive diffusion component for several reasons. Firstly, unlike glucose, glycerol is known to cross biological membranes by passive diffusion (4, 21), making it likely that one of the transport modes in the case of biphasic kinetics reflects passive diffusion. Secondly, the kinetics could not be fitted to a Michaelis-Menton model for two saturable systems (data not shown), but it did satisfy the model for one passive diffusion component and one saturable system. Lastly, the near-vertical nature of the low-affinity component of the Eadie-Hofstee plot strongly suggests that a protein is not involved (21, 30).

A role in glycerol transport may not be the only function of *FPS1*, since deletion of the gene affects cellular lipid composition and results in lower apparent membrane permeability than in the wild type. Likewise, multiple functions for other MIP proteins have been suggested (20). It has also been reported that interaction between the *E. coli* GlpF glycerol transport system and glycerol kinase results in increased activity of the kinase (37), and there are indications that a similar mechanism may be operative in *Xenopus* oocytes expressing the frog lens protein, another MIP family member (20). It may be

possible, therefore, that Fps1p interacts similarly with other proteins.

In addition to the Fps1p-dependent facilitated diffusion system, a distinctive glucose-repressible proton symport for glycerol operates in derepressed cells. The facilitated diffusion system seems to have a function in osmotic regulation (24), whereas the active system probably has a metabolic role since it is induced by a shift from glycolysis to gluconeogenesis (22).

Recently, other MIP family members with unknown functions have been identified in *S. cerevisiae* (3, 27). Although no members of the MIP family are known to act as secondary active transport proteins, the possibility that one of these encodes the active system cannot be excluded, and this is presently being investigated. Alternatively, one or more of these MIP proteins may catalyze glycerol transport under conditions not yet kinetically investigated.

Glycerol transport proteins in microorganisms include both facilitated diffusion and active systems. *Fusarium oxysporum* (8) and *E. coli* (2) transport glycerol by facilitated diffusion, whereas active transport occurs in *Z. rouxii*, *D. hansenii* and *P. sorbitophila* (1, 21, 36). Since *E. coli* GlpF complements the loss of glycerol transport in the *S. cerevisiae fps1*Δ mutant, a comparison between these two systems is of interest. Equilibration of glycerol across the membrane in *E. coli* is very rapid (15), typical of a channel protein, whereas that of the facilitated diffusion system in *S. cerevisiae* has a lower rate of transport. This seems to indicate that the *S. cerevisiae* system may not act like a classic channel protein in all respects. Not all transport proteins conform to rigid classification, however, as demonstrated by the fact that a fraction of the human GAT-1 γ-aminobutyrate mobile transporter protein functions as cation-permeable channels (7). An interesting similarity between Fps1p and *E. coli* GlpF is their effects on membrane functions other than glycerol transport. A *glpF* mutant of *E. coli* showed reduced permeability towards *o*-nitrophenyl-β-D-galactopyranoside and a smaller degree of membrane perturbation by ethanol and dimethyl sulfoxide than the *glpF*$^+$ strain (32), similar to the effects of *FPS1* deletion on membrane function described in this work.

Other microbial glycerol transport systems thought to be involved in osmoregulation are, like the *S. cerevisiae* facilitated diffusion system, apparently not regulated at the genetic level. These include the active systems of *Z. rouxii*, *P. sorbitophila* and *D. hansenii* (1, 21, 36). In *Z. rouxii* and *D. hansenii*, the coupling of glycerol transport to the sodium gradient across the cell membrane controls the maximum rate of transport (23, 36). In addition, some of these systems are regulated at the protein level. The affinity of the glycerol transport system of *Z. rouxii* is dependent upon the extracellular NaCl concentration (36), and activation of facilitated diffusion in *S. cerevisiae* by hypo-osmotic shock and deactivation by hyperosmotic shock have been implicated (24). By contrast, those systems primarily involved in the catabolism of glycerol are frequently subject to catabolite repression; these include the GlpF protein of *E. coli* (15) and active transport in *S. cerevisiae* (22) and *F. oxysporum* (8).

The knowledge of glycerol transport in *S. cerevisiae* has changed dramatically, from the assumption that passive diffusion is the only mechanism of glycerol uptake in this yeast to the establishment of at least two separate transport systems, since the discovery of the *FPS1* gene. As indicated in this paper and previously (24), however, our knowledge of this subject is still incomplete. Further research efforts will be directed toward identification of the gene encoding the active system and elucidation of the diverse functions and regulation of Fps1p and its homologs in *S. cerevisiae*, as well as in other yeasts.

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